The studies on the cell-to-substrate adhesion in vitro(Dissertation_全文)

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論 文 目 録

主論文

1. 題 目

The studies on the cell-to-substrate adhesion in vitro.

(試験管内における細胞の基質に対する接着性の研究)

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   (軟骨細胞の偽足形成に影響を及ぼす因子について)

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2. Changes in the properties of cell-substrate adhesion during cultivation of chicken fibroblasts in vitro in a serum-free medium.

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主論文 1

THE FACTOR AFFECTING THE SPREADING OF CHONDROCYTES UPON THE INORGANIC SUBSTRATE

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Short title:
Chondrocyte spreading

SUMMARY

The effect of conditioned medium (CM) prepared from mass-cultures of chick embryonic cells was studied on the spreading behavior of chondrocyte derived from sternae of 16-day-old chick embryos.

Freshly dissociated chondrocytes exhibited a quite rounded form, and this shape did not change when they were cultured with fresh medium (Eagle's MEM + 6 % fetal calf serum) in vitro for several days. A non-dialyzable material(s) in CM added into the fresh medium stimulated the formation of pseudopods of chondrocytes, without primarily affecting the synthesis of chondroitin sulfates. Such an activity of CM was not lost after boiling, but it was lost following treatment with proteases. The chondrocytes covered with newly deposited acidmucopolysaccharides were insensitive to the effect of CM, but they became sensitive to form pseudopods after treatment of the cells with chondroitinase.

These results suggest that CM has a macromolecular material(s) to enhance the motility or adhesiveness of chondrocytes.

INTRODUCTION

Animal cells cultured in vitro release such molecules into the medium, as influence cell proliferation (Ichikawa, Pluznik & Sachs, 1966; Rubin, 1966; Austin, McCulloch & Till, 1971;

Stanley, Bradley & Sumner, 1971; Watanabe, 1971), differentiation (White & Hauschka, 1971), or adhesiveness (Lillien, 1968; Takahashi & Okada, 1971). Recently, increasing attention is paid to the action of these molecules, and this will give an opportunity to investigate a molecular mediator of cellular interactions.

Many of cell behaviors as influenced by the "conditioning" factor arise from the multiple and complicated cellular processes. For the analysis of the effects of conditioned medium (CM) on such cell behaviors, we need to find the primary action of the factor on cells. In this respect, we searched the initial change in cell behavior which appears immediately after the treatment of cells with CM, using chondrocytes of chick embryo. The results will demonstrate that the spreading of the cells onto a substrate is encouraged by a relatively short-term incubation in CM, which seems to have a significant role on cell's adhesive behaviors to substrates.

MATERIALS AND METHODS

Cell

Chondrocytes from sterna of 16-day chick embryos were used. Sterna thoroughly cleaned from the adherent tissues were treated with a mixture of 0.25 % trypsin (Difco, 1 : 250) and 0.05 % collagenase (Sigma, Type-1) dissolved in Ca^{++}- and Mg^{++}-free saline (abbreviated to CMF, the composition of which was described in Takeichi & Okada, 1972), for 30 min at 37 °C and

subsequently for 90 min with the refreshed same medium. The pH of this medium was adjusted to 7.6-7.8 by adding 1 N NaOH. The softened tissues were transferred into Eagle MEM supplemented with 6 % fetal calf serum (Gibco), and were dissociated into single cells by flushing through a pipette. The cells were well washed with serum-free MEM with several centrifugations and were finally suspended in it. The contamination of non-cartilage cells into the cell suspension obtained by this procedure was quite negligible (cf. Cahn, Coon & Cahn, 1967).

Conditioned Media

Conditioned medium (CM) was prepared from mass cultures of neural retinae or leg muscles. Neural retinae isolated from 11-day chick embryos were dissociated by trypsin according to Lillien (1968). The cells were cultured with 9 ml of MEM supplemented with 6 % fetal calf serum in plastic Petri dishes (Nunclon, 9 cm in diameter). The cell number per plate was adjusted to correspond to the number of cells obtainable from neural retinae of three eyes. After two days of incubation, the medium was replaced with 9 ml of serum-free MEM. After 24 hours, the medium was discarded and the same amount of fresh serum-free MEM was added. During further 4 days, the medium was daily replaced with fresh serum-free MEM. All the medium exposed to the cells for each 24-hour's period during these 4 days was pooled and was used as the medium conditioned with retina (RCM).

Culture of cells of leg muscles from 11-day chick embryos

was prepared using MEM supplemented with 10 % calf serum (Biken), according to Takahashi & Okada (1970). When the cells were grown to a confluence in glass Petri dishes (10 cm in diameter), the medium was replaced with 10 ml of serum-free MEM. Thereafter, the medium conditioned with leg muscles (MCM) was obtained, according to the same schedule as in the collection of RCM.

The pooled CM was asceptically centrifuged at 7,500 x g for 30 min, to remove cell debris and stored in a refrigerater until use.

Culture of Chondrocytes

The effect of CM on cell behavior was tested by observing chondrocytes plated to Falcon plastic dishes (3.3 cm in diameter). The chondrocytes suspended in serum-free MEM were placed on the surface of the dish in a density of approximately 360 cells per mm^2. After 15 min at 37 °C, most of the inoculated cells were firmly attached to the dish, and then the fluid was replaced with 1.0-1.5 ml of a medium, the biological activity of which will be assayed. As the standard culture medium (to be called "fresh medium") for chondrocytes, MEM supplemented with 5-6 % fetal calf serum was generally used. The cells were incubated at 37 °C in 5 % CO_2 in a water-saturated atmosphere.

For suspension culture, 1 x 10^6 of chondrocytes were inoculated into a hydrophobic plastic Petri dish (Jintan-Terumo, 10 cm in diameter) with 10 ml of medium. Most of the inoculated cells were maintained as a suspension at least for two days in

this Petri dish.

Criteria for comparing cell morphology

The observation of cells was carried out using a phase-contrast microscope in a dark contrast field. The cells which appear refractile and rounded were identified as "non-spreading", whereas the cells which appear dark and flattened with pseudopodial processes, as "spreading" (cf. Fig. 4-7).

The degree of cell spreading was represented by a ratio of spreading cells in a viable cell population. The cell counts were performed in randomly-selected several fields of culture plates subjected to a same experimental condition. Usually 120-200 cells were counted per field. The cell viability was tested with Trypan blue staining, and it was found that more than 90 % of attaching cells were viable in any present experimental condition.

Labeling of cells with $S^{35}O_4$

The chondrocytes were pulse-labeled for 2 hours with the incubation in the labeling medium (Eagle MEM without "cold" sulfate supplemented with 6 % fetal calf serum which had previously been dialyzed against sulfate-free Hanks' solution) containing 10 μc/ml of sodium sulfate-S^{35} (625 mC/mM, New England Nuclear Corp.). After labeling, the cells were rinsed with Hanks' solution and then chased for 1 hour in the above

medium containing "cold" sodium sulfate in concentration 300 times greater than the labeled sulfate.

The labeled plates were rinsed several times with Hanks' solution and fixed for 10 min in 2.5 % glutaraldehyde. After repeated washings with saline, the plates were rinsed with ethanol (with 70 % and subsequently 100 %), and air-dried. The cells were covered with liquid emulsion (Konishiroku Photo Ind. Co., NR-M2) and exposed for a week in a refrigerator. The emulsion was developed in Konidol-X (Konishiroku Photo Ind. Co.).

RESULTS

Effect of conditioned medium on cellular morphology

Freshly-dissociated chondrocytes were very homogenous in their morphology and all were with rounded form. The cells attached to the plate were incubated for 24 hours using fresh or conditioned medium, supplemented with 6 % fetal calf serum respectively. At the end of the incubation, a conspicuous difference in cell morphology appeared between cells in the fresh and those in the conditioned medium (Fig. 4, 5). In the fresh medium, a large number of cells remained round, whereas the cells in CM spread out onto the surface of culture dish by extending pseudopods (Table 1). Each specific appearance of cells were maintained for further prolonged incubation, although the cells started to proliferate. The round cells in the fresh

medium tended to be gradually detached from the substrate after several-days, while the spreading cells remained attached. Both RCM and MCM showed the same effect.

The appearance of the observed difference in cell morphology between cells in different media has not been caused by a selective overgrowth of a particular cell type in either medium, because the tested culture period was too short to expect such a possibility.

Partial characterization of the active factors in CM

To estimate a molecular nature of factor(s) to induce the spreading of chondrocytes, a sample of CM was dialyzed by cellulose tubing (Visking Co.) against 100-fold amount of MEM for 24 hours, and another sample was filtered through a collodione bag (Carl Schlicher and Schull) by aspiration. Both macromolecular fraction obtained by dialysis and low-molecular fraction by ultrafiltration of CM were assayed to see which fraction has a spreading-promoting activity. As shown in Table 1, the activity was retained in the dialyzed CM, although it was slightly reduced, while the ultrafiltrate had no activity. Since both RCM and MCM had ~~an~~ almost the same activity, the former ~~one~~ was exclusively used for the later assays.

The heat-stability of the active factor(s) in CM was tested. The ten-fold concentrated RCM by collodione bag was dialyzed against CMF and treated at 60 °C for 20 min using a water bath.

Another sample of the concentrated and dialyzed RCM was treated in boiling water for 10-15 min. Both of the treated CM were each mixed with fresh medium in various concentrations and its activity was tested. The treatment of CM with 60 °C did not reduce the activity. The boiling also did not, but it rather raised the activity, as shown in Fig. 1. These results show that the effective factor(s) in CM is very stable against heating.

RCM was treated with two kinds of proteases. The concentrated and dialyzed RCM was mixed with 0.1 % crystallized-trypsin (Sigma Type 1, 2 x crystallized) dissolved in CMF in a ratio of 8 : 1. As a control of the trypsin action, 1/10 part of 0.67 % trypsin inhibitor (Sigma Type 1-S, from soybean) in CMF was added in the RCM-trypsin mixture, prior to the addition of the trypsin. The same part of CMF was also added in the above experimental mixture to adjust the concentration of the constituents. As another control, RCM containing trypsin inhibitor only was prepared. These solutions were incubated at 37 °C for 60 min. After the treatment, they were incubated in boiling water for 15 min to inactivate the enzyme. These treated mixtures were added to the fresh medium in 10 % of concentration and the activity was tested. Table 2 shows that the activity of CM was significantly reduced following treatment with trypsin.

The effect of pronase on the activity of RCM was tested. 0.05 % of pronase (Kaken-Kagaku Co., 45,000 P.U.K./g) in CMF was mixed with the concentrated and dialyzed RCM in a ratio of 1 : 9

and the mixture was incubated at 37 °C for 60 min. For inactivating the pronase, the solution was further treated in boiling water for 15 min. As the control, the previously-boiled pronase was added into boiled CM. The activity of RCM was completely lost following treatment with pronase (Table 2). These results suggest the protein-nature of the active factor(s) in RCM.

Acquisition of spreading property of chondrocytes in CM

On the causes of the spreading behavior of chondrocytes in CM, two possibilities would be considered. One is that the active macromolecules in CM adsorbed on the surface of culture dish may increase the contact affinity of cells to the substrate, so that the cells can easily spread on it. This occurred in fact in the case of the attachment of fibroblastic cells to a plastic surface coated with a substance in CM (Takeichi, 1971). Another is that a motile or adhesive property of cells themselves may be altered by CM. To determine which is the case, the following experiments were performed.

The chondrocytes were cultured at a suspended state with the fresh or conditioned medium. The latter is composed of 1 part of the boiled RCM (concentrated ten-fold as described in the previous section) and 9 part of the fresh medium. The control fresh medium contains 10 % of CMF instead of the RCM. When the cells were cultured for 2 days, there were small

aggregates and singly dissociated cells in each medium. They all were with rounded form. These cells were collected by centrifugation and washed once with serum-free MEM, and finally suspended in it. They were plated in Falcon plastic dish with the same method as used for the freshly-dissociated cells, and subsequently cultured with the fresh medium.

The cells or cell aggregates pre-cultured in CM was observed to spread rapidly after attaching to the substrate and more than 80 % of the inoculated cells spread by several-hour's incubations (Fig. 2 and 6, 7). Most of the cells pre-cultured in the fresh medium did not spread during these incubations. This result indicates that the cell property itself of the chondrocytes was converted by the pretreatment with CM to extend easily on the substrate, and it may exclude the possibility of the alternation of the substrate property by adsorption of CM as the cause for permitting the chondrocytes spreading.

Inhibition of the action of CM by accumulation of chondroitin sulfates over cell surfaces

As shown in Table 3, the chondrocytes previously cultured in vitro (for 2 days in the fresh medium) became much less sensitive to the spreading-enhancing activity of RCM than the freshly dissociated chondrocytes. Since the cells, as revealed by staining with toluidine blue, secrete acidmucopolysaccharide over cell surfaces (cf. also Kojima & Yamagata, 1971), during the culture period, it seems probable that such molecules will

render the cells less sensitive. Thus, it was tested, if the removal of the acidmucopolysaccharides from cell surfaces may convert the cells to be sensitive to CM to spread.

The chondrocytes cultured for 2 days in fresh medium were treated with 1 unit/ml of purified chondroitinase AC (Seikagaku Kogyo Co., prepared from Flavobacterium heparinum) dissolved in Hanks' solution buffered with 0.01 M Hepes to pH 7.2, at 37 °C for 60 min without detachment of the cells from the substrate. The control cultures were treated with the enzyme-free same Hanks' solution. After the treatment, the cells were rinsed several times with ~~the~~ Hanks' solution and incubated with fresh or conditioned medium for 24 hours. It was found that this treatment was clearly effective for the cells to restore their sensitivity to CM to spread (Table 3). It is therefore considered that the accumulation of chondroitin sulfate or the other chondroitinase-sensitive acidmucopolysaccharides over the cells prevents the action of CM.

Influence of CM on the synthesis of chondroitin sulfate

It has been often reported that the chondrocytes with a fibroblastic appearance in cell culture show a lower synthetic activity of chondroitin sulfate (Abott & Holtzer, 1966; Coon, 1966; Bryan, 1968). To determine whether the cessation of the synthesis of chondroitin sulfate is a prerequisite for the spreading of cells, the chondrocytes which had previously been

cultured for 24 hours in fresh or conditioned medium were pulse-labeled with $S^{35}O_4$. It can be assumed that the the bulk of $S^{35}O_4$ label in chondrocytes is incorporated into chondroitin sulfate (Cahn & Lasher, 1967; Nameroff & Holtzer, 1967).

Most of the round cells in fresh or conditioned medium were labeled with $S^{35}O_4$. The grains were scattered over cell surfaces and cell peripheries. The density of grains per cell was varied with each cell; some were intensely labeled, the other weakly, in spite of their common round morphology. In the case of flattened spreading cells cultured in CM, the cells exhibiting a typical fibroblastic shape, with a elongated pseudopodial processes, were always labeled weakly as was previously reported. The flattened polygonal cells, however, were labeled sometimes heavily as much as the round cells (Fig. 3 and 8, 9). This demonstrates that the cessation of synthesis of chondroitin sulfate is not always a direct cause for the cell spreading in CM.

DISCUSSION

The present studies demonstrated that some types of cultured cells release the macromolecular substance with the spreading enhancing activity into the culture medium. The active substance contained in the conditioned medium is probably a protein or a protein-complex, since its activity is lost ~~following~~ (by the) treatment with proteases. The observed increase in the activity after boiling

treatment might be due to the inactivation of some inhibiting molecule. For more precise characterization, the purification of the active molecule will be required.

As to the mechanisms how the active factor influence the spreading behavior of chondrocyte, two possibilities will be considered. The first is that CM increases the cell adhesiveness to substrates. Since the polyanions like acidmucopolysaccharides has been known to be potent inhibitors for cell adhesion (Nordling, Vaheri, Saxén & Penttinen, 1965), the cell adhesiveness will be increased by CM, if the latter were to act to remove chondroitin sulfate on the surfaces of chondrocytes. But, the present results exclude such activity of CM, because CM does not exert its effect for cell spreading, as long as the cells were covered with chondroitin sulfate, and the use of chondroitinase was necessary to render the cells to be responsive to CM. It was also examined, if CM may inhibit the synthesis of chondroitin sulfate and thus it leads to an increase of cell adhesiveness. The results do not give any evidence in favor of this possibility, too.

The second possibility is that the motility of the cell is increased by the factor(s) contained in CM. Since the cell spreading by the protrusion of pseudopods is based on the active movement of cytoplasm, the increase in the motility of cells would ease the spreading on the substrate. Some evidences indicating a significant role of cell's motility in the cell

adhesion to the substrate have recently been given (Takeichi et al. 1972). The cells can increase actively the contact area between the apposed surfaces of cell and substrate by spreading, and consequently a stronger adhesion will be attained (see Curtis, 1967, on the discussion of this problem).

Chondrocytes showed the same response to CM prepared with either neural retina or leg muscle cells. According to our another observations, the medium conditioned with pigment epithelium or sclera cells had the same activity as reported here (unpublished data). It seemes therefore that the cultured embryonic cells generally release the factor(s) to spread the chondrocytes in culture. The question will still remain whether the described effect of CM is specific for chondrocyte, or general for cells derived from various tissues. The study of this problem is in progress.

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Table 1. Effects of CM on the spreading of chondrocytes.

Media	% spread cells		
	Expt.1	Expt.2	Expt.3
Fresh medium	23.7 ± 0.1	2.6 ± 0.4	27.1 ± 3.9
RCM	73.4 ± 1.6	86.1 ± 1.2	
MCM	78.1 ± 3.1		81.6 ± 1.3
Dialyzed RCM	63.8 ± 2.5	69.1 ± 1.3	
Dialyzed MCM	74.7 ± 2.6		72.8 ± 2.2
RCM-filtrate		3.1 ± 0.6	

The data represent the means of cell counts in three (in Expt.1) or four (in Expt.2 & 3) different fields with S.E.

Table 2. Effect of proteases on the activity of RCM

Media		% spread cells
Fresh medium		11.7 ± 2.1
RCM		66.3 ± 2.3
Trypsin	+ RCM	20.8 ± 1.9
Trypsin + T.I.	+ RCM	56.6 ± 4.8
T.I.	+ RCM	64.5 ± 2.4
Pronase	+ RCM	11.6 ± 3.2
Inactivated pronase	+ RCM	64.8 ± 1.1

The control RCM was also treated with boiling water. Trypsin inhibitor was abbreviated to T.I. The data represent the means of cell counts in four different fields with S.E.

Table 3. Effect of RCM on the spreading of the pre-cultured chondrocytes.

Media	% spread cells
Expt.1	
Fresh medium	1.4 ± 0.6
RCM	5.0 ± 2.6
Expt.2	
Fresh medium	7.0 ± 0.6
RCM	23.7 ± 1.9
CHase---Fresh medium	12.3 ± 1.5
CHase---RCM	63.0 ± 5.9
Before the treatment	7.6 ± 0.4

Chondroitinase AC was abbreviated to CHase. The data represent the means of cell counts in four different fields with S.E.

FIGURE LEGENDS

Fig. 1. Effect of boiling treatment on the activity of RCM. The treated or non-treated RCM which had previously been concentrated ten fold was respectively added into the fresh medium in various concentrations as indicated at the abscissa. The assay was performed at 24 hours after inoculation of cells. Each plot represents the mean of cell counts in three different fields with S.E. ○, treated; ●, non-treated.

Fig. 2. Rate of the spreading of chondrocytes attached to the substrate after suspension culture. The abscissa is the time after inoculation of cells. The cells were cultured in a serum-free MEM for first 15 min, for sticking them to the substrate. Each plot represents the mean of cell counts in three different fields with S.E. ●, pre-culture with fresh medium; ○, pre-cultured with RCM.

Fig. 3. Distribution of differentially labeled chondrocytes. The ordinate is the percentage of the cells, having grains in such number as grouped at the abscissa, to the total counted cells. Rounded cells in fresh medium and spread cells in RCM were counted respectively. In the latter case, the cells were divided into two cell types, the elongated "fibroblastic" one and the polygonally flattened one.

The total number of counted cells was 125 in the case of fresh medium and 164 in that of RCM. The counts were made to the grains both over the cells and at the extracellular portion close to the cell periphery. ▬▬, rounded cells; ▨, elongated cells; ▭, polygonal cells.

Fig. 4, 5. Phase-contrast micrographs of chondrocytes cultured for 24 hours in fresh medium and RCM respectively. X 180.

Fig. 6, 7. Phase-contrast micrographs of chondrocytes attached to the substrate after suspension culture. Photographed at 180 min after inoculation. Arrow indicates a spreading cell aggregate. Fig.6, pre-cultured with fresh medium; Fig.7, pre-cultured with RCM. X 180.

Fig. 8, 9. Autoradiographs of chondrocytes labeled with $S^{35}O_4$, cultured with fresh medium and RCM, respectively. X 710.

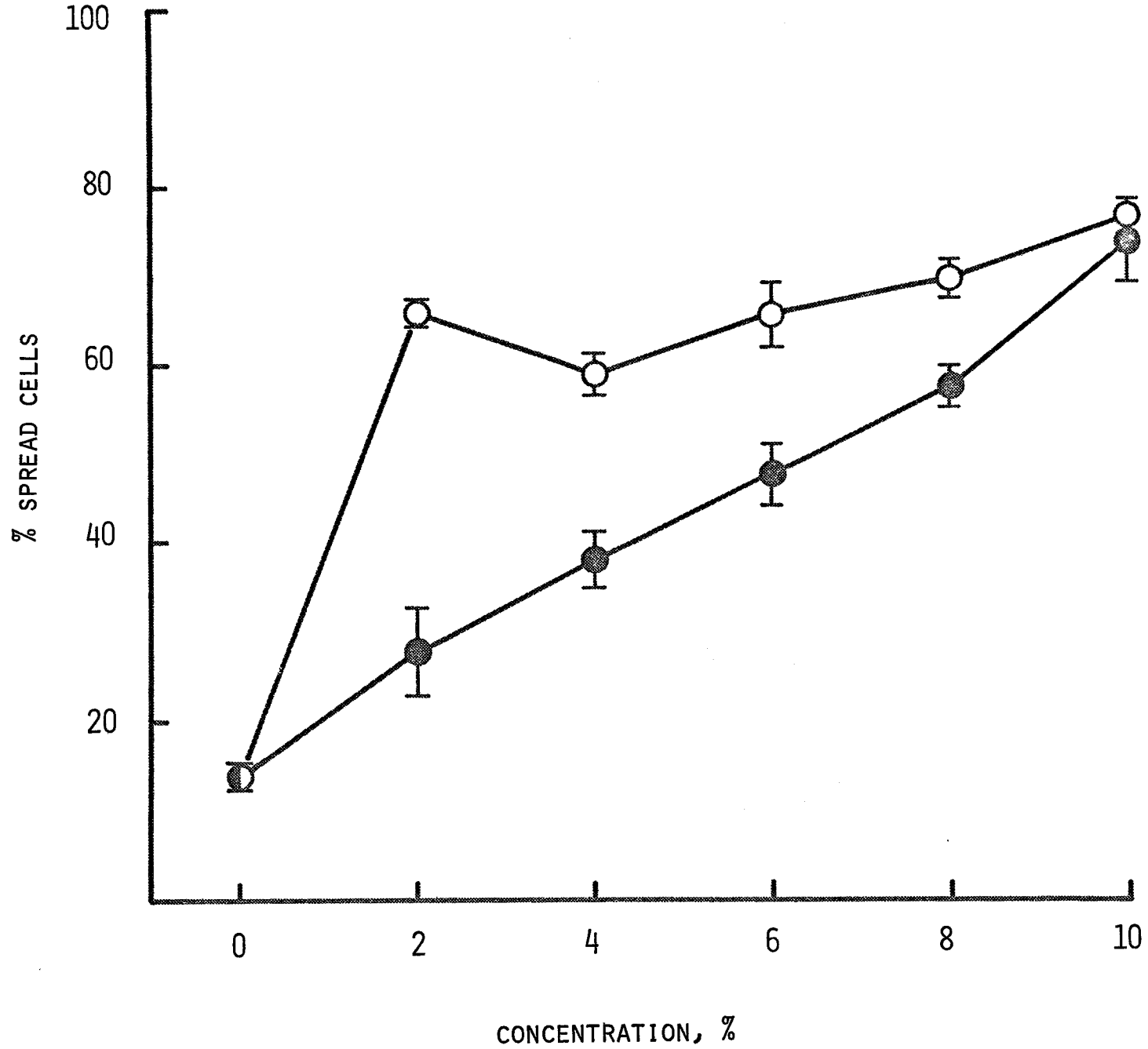
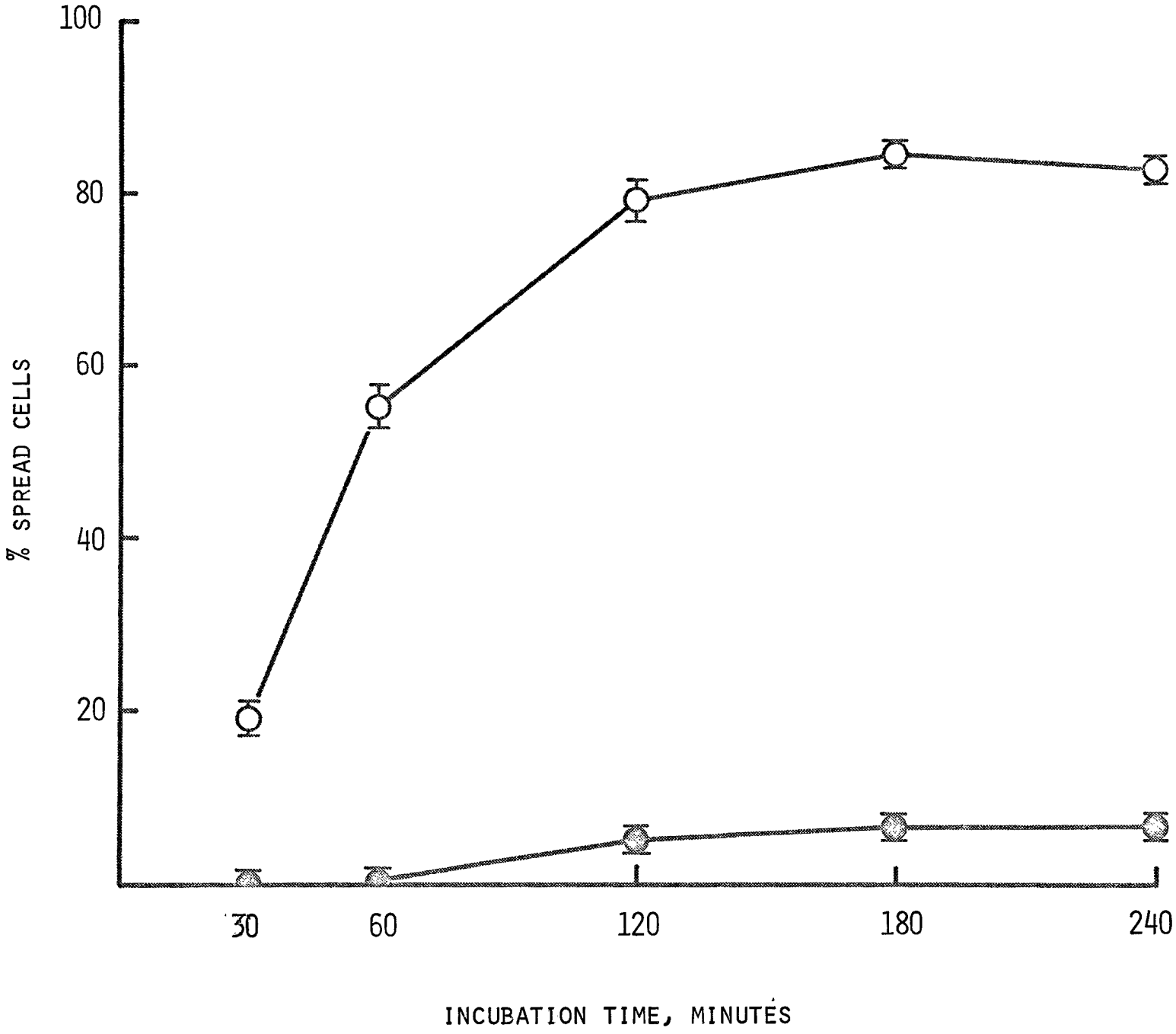


Fig. 1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80
60
40
20
% SPREAD CELLS
30
60
120
180
240
INCUBATION TIME, MINUTES

Fig 2

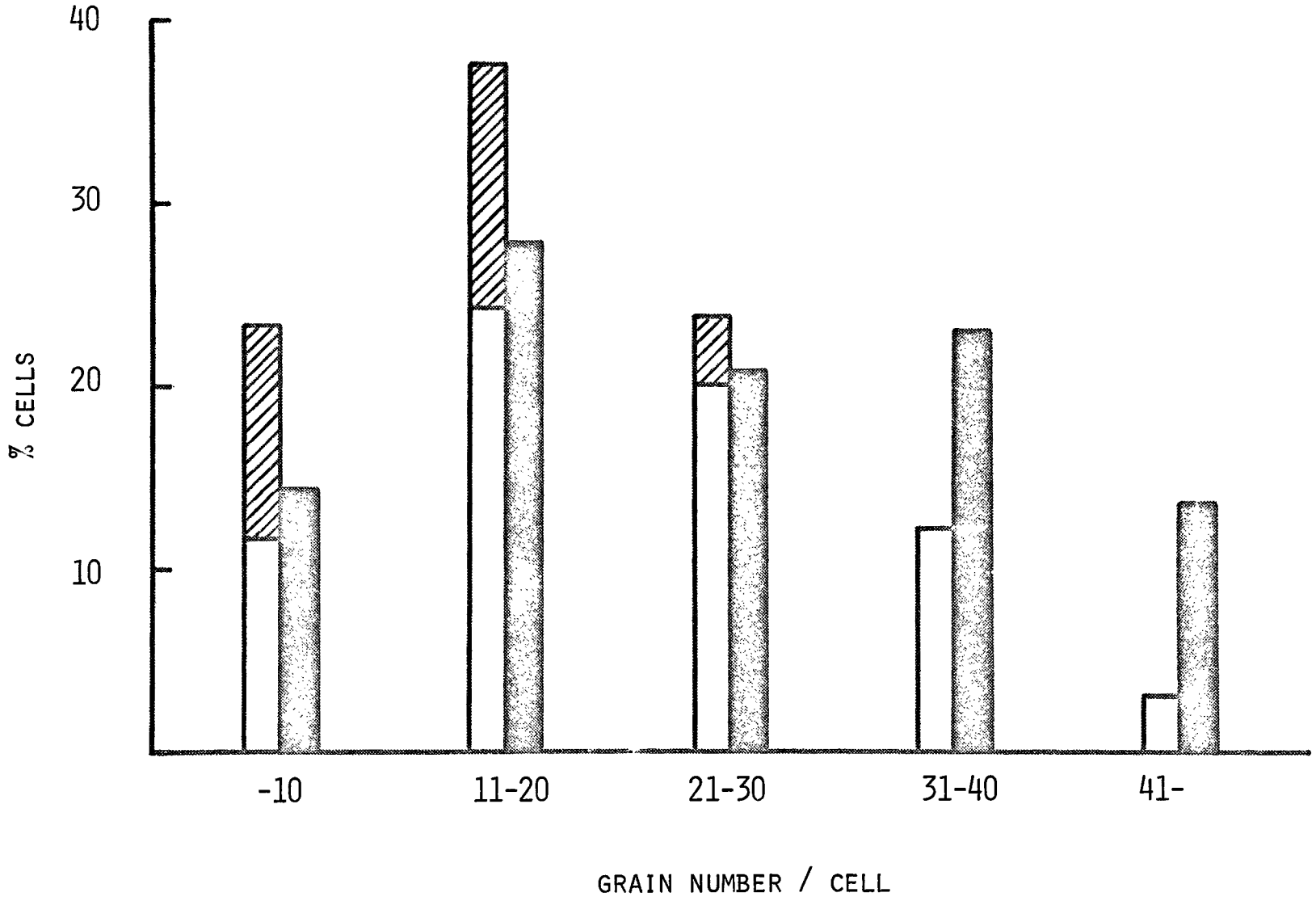

Fig 3

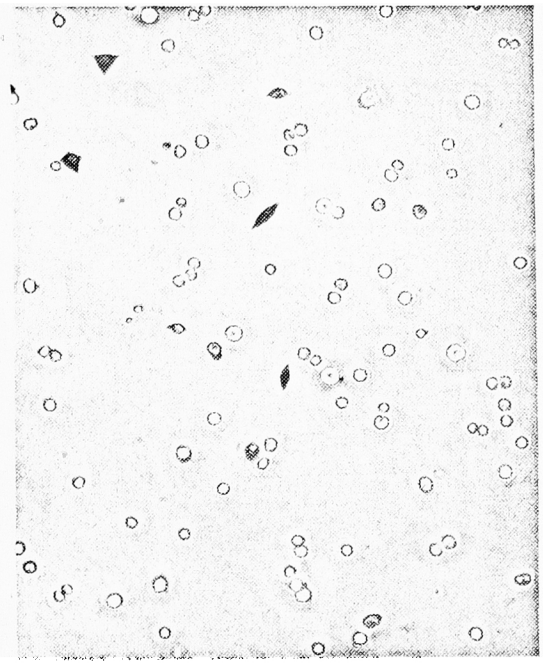

Fig. 4

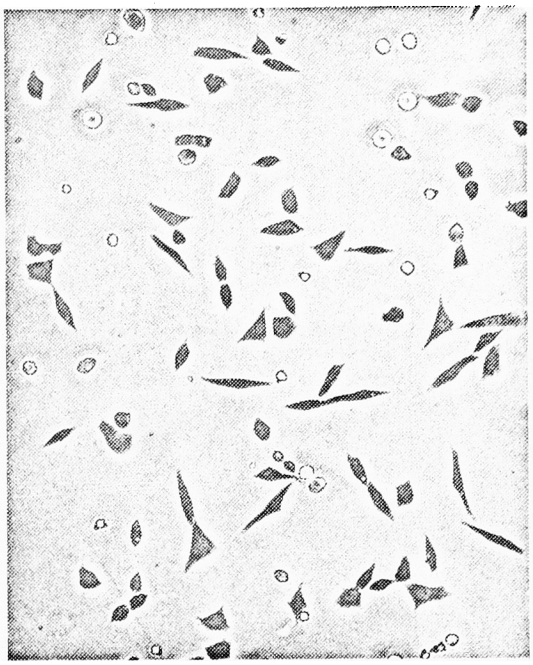

Fig. 5

Fig. 6

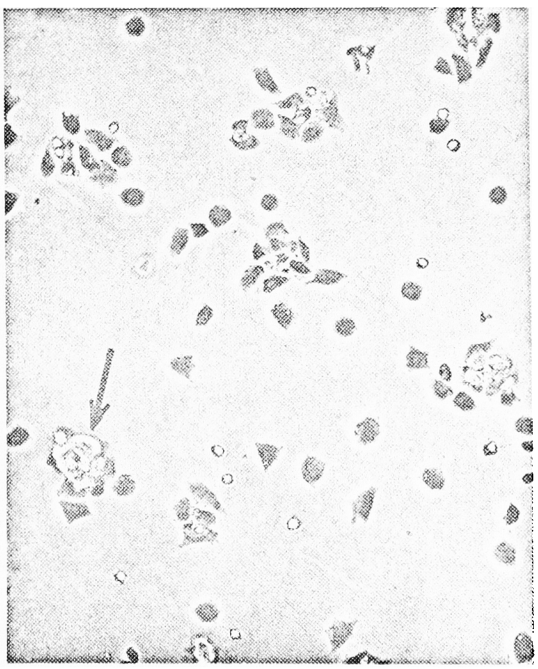

Fig. 7

Fig. 4 – 7

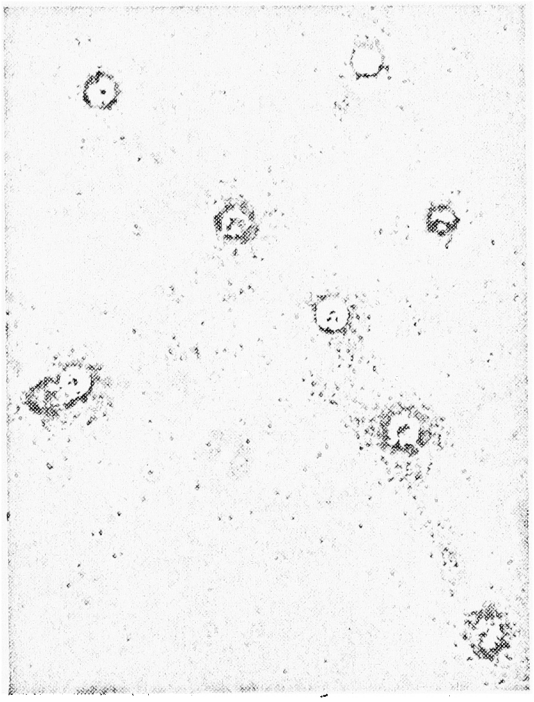

Fig. 8

Fig. 9

Fig. 8 & 9